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*Original Contribution*

# SERTOLI CELL-ENRICHED FRACTIONS IN SUCCESSFUL ISLET CELL TRANSPLANTATION

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☐ **Abstract — Prolonged survival of islet- allo- and xenografts can be induced following implantation of the islets into the abdominal testis of diabetic rats. We previously showed that a factor released by Sertoli cells appears to be responsible for the protection of the intratesticular islet allo- and xenografts against rejection. The aim of this study was to examine whether an immunologically privileged site can be established in an organ site in vivo, other than the testis, such as the renal, subcapsular space, to make feasible the grafting of female recipients as well. A total of 36 male and 21 female, diabetic, PVG rats were divided into six different treatment groups: 1) Six male rats were grafted with islets from Sprague-Dawley (S-D) donor rats only. 2) Ten male rats were grafted with islets from (S-D) donors and were then given a short course of cyclosporine (CsA) posttransplantation. 3) Ten male rats were grafted with islets from (S-D) donors and with Sertoli cell-enriched fractions (SEF) from PVG donors but without CsA. 4) Ten male rats were grafted with a combination of islets from (S-D) and SEF from (PVG), donors, respectively, and CsA. 5) Ten female rats were given an identical combination of cells and CsA as depicted for group 5. 6) Ten female rats were grafted with a combination f islets and SEF, both cell types from S-D donors, and CsA. The results showed that 70% to 100% of the grafted rats in groups 1, 2, and 3 remained hyperglycemic. Prolonged normoglycemia in excess of 100 days was induced in more than 75% of the grafted rats only in groups 4, 5, and 6, or in those animals who were grafted with a combination of islets and SEF and who were given a short course of CsA as well. Electron microscopic examination of the grafted tissues showed the presence of intact beta cells and of cells with features characteristic of Sertoli cells. Our results suggest that 1) the protection of islet allografts in nonimmunologically privileged site can be achieved in male and female rats by means of the simultaneous transplantation of Sertoli cells. 2) Sertoli cells apparently maintain the capacity to secrete an immune inhibitor in organ sites other than the testis. We conclude that it is feasible to create an immunologically privileged site for the transplantation of isolated islets in male and female diabetic recipients without the need for sustained immunosuppression.**

☐ **Keywords — Sertoli; Allograft; Immunologically privileged.**

## INTRODUCTION

Several immunologically privileged sites in mammals sometimes allows prolonged survival of transplanted allografts (1). The immunologically protective mechanisms of the brain and anterior chamber of the eye seem to involve primarily deficient lymphatic drainage (2). Indeed, in some instances, experimental interruption of lymphatic drainage in tissue has created an immunologically privileged site (3). In contrast, the testis (also a privileged site), has excellent lymphatic drainage (4). It is likely that locally produced factors are responsible for inhibition of the immune response (5).

We previously showed that extended survival of islet allo- and xenografts can be achieved after transplantation of isolated pancreatic islet cells into the abdominal testis (6–8). The donor origin of these isolated cells does not seem to influence their long-term survival. Islet cells grafted against major histocompatibility (MHC) barriers (6), islet xenografts (7), and islets of MHC-compatible donors grafted into the testes of rats with autoimmune, spontaneous diabetes mellitus function indefinitely in diabetic recipients (8). However, despite remarkable preservation of function in abdominal, intratesticular islet allografts, there are drawbacks associated with the use of an unconventional organ site. A major concern is that the germ cells may undergo a malignant transformation at the higher core body temperature (9). More importantly, because

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only males have Sertoli cells, this transplantation approach cannot be used in that half of the population which is female.

Because of these objections, and because of the observations that cultured Sertoli cells produce a factor which inhibits the production of IL-2 in vitro (10), studies were initiated in an attempt to create an immunologically privileged site in a heterotopic site, in vivo, other than the testis. In the present study we investigated the effect of Sertoli cell-enriched fractions (SEF) transplanted in conjunction with isolated islets on islet allograft survival in the renal subcapsular space of diabetic rats.

## MATERIALS AND METHODS

### *Animals*

PVG rats, weighing between 150–200 g, were used exclusively as recipients of islets. Diabetes was induced by means of a single i.v. injection of 65 mg/dL of streptozotocin. Only rats with plasma glucose levels in excess of 400 mg/dL were transplanted. Sprague–Dawley (S–D) outbred rats were used as islet donors. Either PVG or S–D male rats between 16 and 18 days old were used as Sertoli cell donors.

### *Islet Preparation*

Islets were prepared according to modification of the method of London et al. (11), described in detail elsewhere (6). The islets were purified on Ficoll gradients, and the isolated cells were then incubated for 4 days at 37°C in a humidified atmosphere of 5% $CO_2$ and air prior to use (6). No special efforts were made to deplete the islets of contaminating passenger leukocytes.

### *Sertoli Cell-enriched Fraction Preparation*

Highly purified preparations of Sertoli cells were isolated from the testes of young males according to the method of Cheng et al. (12). The testes were removed, chopped into several pieces, and placed in a 50 mL conical tube containing 50 mL of Ham's F12/DMEM media. The pieces were washed once by centrifugation at 800 × *g* for 2 min. The supernatant was aspirated, and the tissue resuspended in 40 mL of media containing 40 mg trypsin and 0.8 mg DNase in a sterile 250 mL Erlenmeyer flask. The flask was placed in an 37°C oscillating incubator at 60–90 osc/min for 30 min. This step removed Leydig cells. The tubules were then transferred to a 50 mL conical tube, and centrifuged at 800 × *g* for 2 min. The supernatant fraction was aspirated, and the pellet resuspended in 40 mL of 1 M glycine, 2 mM EDTA containing 0.01% soy bean trypsin inhibitor and 0.8 mg DNase, and incubated at room temperature for 10 min. This step lysed any residual Leydig cells. The cells were washed by centrifugation for 2 min, and the step repeated twice, or until the media was no longer cloudy. The pellet was resuspended by gentle homogenization with a glass Pasteur pipet in 40 mL of media containing 20 mg collagenase in an Erlenmeyer flask, and incubated at 37°C for 5 min with 60–90 osc/min. The cell suspension was centrifuged at 800 × *g* for two min, and the pellet resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg collagenase and 0.2 mg DNase, and incubated in an Erlenmeyer flask at 37°C for 30 min with 60–90 osc/min. The cells were then washed by centrifugation for 2 min, and the process repeated at least three times to eliminate peritubular cells. The cells were resuspended by gentle homogenization with a Pasteur pipet in 40 mL media containing 40 mg hyaluronidase and 0.2 mg of DNase, and incubated at 37°C for 30 min with 60–90 osc/min. The cells were pelleted by soft centrifugation for 2 min, and washed at least five times to eliminate germ cells. The resultant SEF was resuspended in 0.25 mL of media, and immediately transplanted into the recipient rat. Each grafted rat received the equivalent of the total amount of Sertoli cells contained in a single testis.

### *Transplantation of Rats*

The diabetic rat was anesthetized with methoxyflurane USP in a sterile hood and the left flank opened to expose the kidney. The SEF was injected first underneath the renal capsule. The cells could be seen as a milkish bubble underneath the capsule. Immediately afterwards, a total of 10 islets/g of body weight was injected into the same milkish bubble. The needle was retracted slowly to prevent leakage of the grafted cells. Cyclosporine (CsA) was administered s.c. in varying doses over a 20-day period to groups two and four. Because the grafted rats responded similarly whether the drug was administered over a 20-day, or over a 3-day period, all of the subsequent groups, including the female rats, were treated with only three injections of 25 mg/kg CsA, given on days 0, +1, and +2, relative to the graft. The rats received no other therapy.

A total of 36 male and 21 female PVG rats were divided into six different treatment groups: *Group 1*, the control group, consisted of 6 male rats grafted with only islets from S–D donor rats. They received neither SEF nor CsA. *Group 2* consisted of 10 rats grafted with a combination of islets from S–D rats and CsA posttransplantation, but no SEF. *Group 3* consisted of a total of 10 rats grafted with a combination of islets from S–D and SEF from PVG donor rats, but no CsA posttransplantation. *Group 4* consisted of 10 rats grafted with a combination of islets from S–D donors, SEF from PVG donors, and CsA posttransplantation. *Group 5* consisted of 11 female rats grafted with the same combination of cells as depicted for Group four. *Group 6* consisted of 10 female rats grafted with a

combination of islets and SEF, both cell types from S-D donors, and CsA posttransplantation.

*Posttransplantation Evaluation of Rats*

The grafted rats were transferred to metabolic cages, and plasma glucose levels were obtained at weekly intervals. Urine volumes and urine glucose contents were obtained at daily intervals. A rat was considered cured of the diabetic process if the following criteria were met: A random plasma glucose level ≤ 150 mg/DL; aglycosuria; and immediate reversal to hyperglycemia following surgical removal of the grafted kidney.

To determine if any of the rats had become unresponsive to their grafts, normoglycemic rats were challenged with a secondary islet allograft consisting of at least 500, freshly prepared, Sprague–Dawley islets which were injected into the contralateral renal subcapsular space. No immunosuppression was given following the challenge.

To examine the impact of the transplantation of SEF on fertility of the female rats, normoglycemic animals of longer than 30 days were mated with PVG males. Metabolic parameters, as outlined above, were closely monitored, as was the course of their pregnancies.

*Structural Analysis of Grafted Tissue*

A total of five successfully grafted rats were nephrectomized at intervals following transplantation. Wedge sections of renal tissue, obtained from sites at which islets and SEF had been injected, were prepared for examination by light and electron microscopy, as previously described (15). Briefly, the tissue wedges were immersion-fixed with 5% glutaraldehyde in 0.1 M s-Collidine buffer for 1 h, washed in buffer, and post-fixed for 1 h with 1% osmium tetroxide in 0.1 M buffer. Small tissue blocks were cut from the wedges, and dehydrated through a graded series of ethyl alcohols, transferred to propylene oxide, and embedded in Epon 812/Araldite plastic resin. Thick (0.5 $\mu$m) and thin (900 ng) sections were stained routinely with toluidine blue and uranil acetate/lead citrate, respectively, for structural analysis by light and electron microscopy.

## RESULTS

The results are summarized in Table 1:

*Group 1:* None of the six rats grafted with islets alone, without either SEF or CsA, became normoglycemic.

*Group 2:* Three of 10 rats grafted with islets and treated with CsA became normoglycemic for more than 100 days. The 3 normoglycemic rats were challenged with a secondary graft on days 116, 192, and 197, respectively. One rat reverted to hyperglycemia on day 130, while 2 remained normoglycemic.

*Group 3:* Initially 6 of the 10 rats grafted with islets and SEF, but no CsA, became normoglycemic, but all of them reverted to hyperglycemia by day 14.

*Group 4:* All 10 of rats grafted with a combination of SEF and islets, and also given CsA became normoglycemic. Two reverted spontaneously to diabetes on days 19 and 76, respectively. Three were nephrectomized on days 58, 84, and 167 following transplantation. All 3 of these rats became hyperglycemic within the next 24 h. The remaining 5 rats were challenged with a secondary islet allograft on days 119, 129, 280, 342, and 400, respectively. Of these, the first 2 reverted to diabetes on day 127 and 139, respectively, while the latter 3 remained normoglycemic.

*Group 5:* All 11 of the female rats grafted with a combination of islets and SEF, and then given CsA, became normoglycemic. Of these, 4 reverted spontaneously to hyperglycemia by day 28. Of the 7 normoglycemic rats who were mated with male PVG rats, 6 became pregnant, and of these, 8 had litters varying between 1 and 10 pups. They were able to nurse the pups successfully. A total of 7 of the long-term surviving females were challenged with secondary islet allografts at least 200 days following transplantation. None of them reverted to hyperglycemia.

Table 1. Effect of Sertoli cells on islet allograft survival in the non-immunologically privileged renal, subcapsular site

| Group (*n*) | Gender | Sertoli cell (donor origin) | CsA | Duration of normoglycemia (days) Individual responses |
|---|---|---|---|---|
| 1 (6) | male | – | – | 0,0,0,0,0,0 |
| 2 (10) | male | – | + | 0,0,0,0,0,0,0,130,>441,>445 |
| 3 (10) | male | +(PVG) | – | 0,0,0,0,9,10,12,13,13,14 |
| 4 (10) | male | +(PVG) | + | 19,76,58*,84*,167*,127†,139†,>418†,>422†,>425† |
| 5 (11) | female | +(PVG) | + | 7,11,14,28,>287†,>305†,>306†,>308†,>441†,>447†,>457† |
| 6 (10) | female | +(S-D) | + | 8,10,96*,128*,>168,>172,>184,>193,>193,>196 |

*nephrectomized, †challenged with a secondary islet allograft.

*Group 6:* Of the 10 rats grafted with islets and SEF from the same donor strain of rat, all 10 became normoglycemic. Two reverted to hyperglycemia by day 10. A nephrectomy to remove the graft was done on 2 of the long-term surviving rats on days 96 and 201, respectively. Both reverted to hyperglycemic immediately within the next 24 h.

*Tissue Morphology*

Renal tissue obtained from the long-term grafted kidney appeared structurally normal by light microscopy (Fig. 1). Transplanted islets in this organ were immediately subjacent to the kidney capsule, and also appeared structurally normal. They displayed tissue and cellular architecture identical to islets in situ (Fig. 1). Individual islet cells were partitioned into cell clusters by thin connective septa containing small vessels and capillaries (Fig. 1). It appeared that most of the islet cells contained secretion granules. When resolved by electron microscopy, islet cells were identified as the $\beta$-cell type by the inclusion of ultrastructurally distinctive, and unique insulin-containing secretion granules (Fig. 2). All $\beta$-cell clusters observed were in close proximity to intra-islet capillaries (Fig. 2).

There was a high density of cells between, and directly adjacent to, the transplanted islets and renal parenchyma. By light microscopy, they did not appear to be islet cells, kidney cells, nor cells of blood origin (Fig. 1). When observed by electron microscopy, these cells were similar in ultrastructure to Sertoli cells in that their nuclei were irregular in profile, and contained deep nuclear clefts, distinctive nucleoli were often present, and mitochondrial structure was dense. Although these cells did not retain the typical polarity of Sertoli cells in vivo, they were, however, identical in appearance to Sertoli cells in vitro, when the cells are not plated on a basement membrane substrate (33,34). The cells were not associated with a basement membrane, and appeared randomly organized (Fig. 3). Cells showing ultrastructural features of either germ or Leydig cells were not observed.

Fig. 1. Normal pancreatic islets of Langerhans (IL), along with isolated rat Sertoli cells, were transplanted into the subcapsular space in the kidney of diabetic rats. As illustrated in this light micrograph, islets appear structurally normal, and are well-vascularized as indicated by the numerous intra-islet capillaries. The kidney parenchyma (K) also appears normal. Between the islet and renal tissue there is a high density of cells (S) which do not appear to be of islet, renal, or blood origin. Tissue was stained with toluidine blue. ×430.

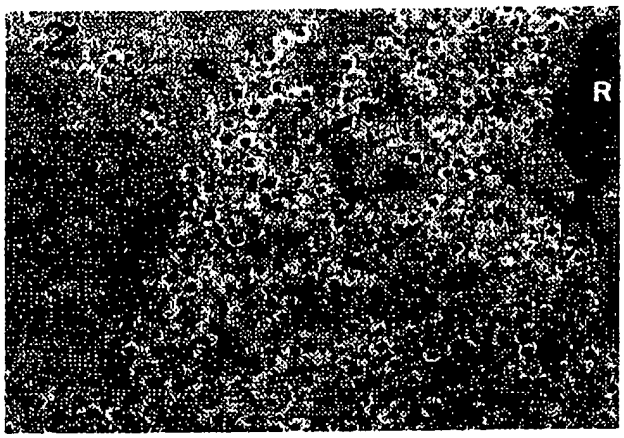


Fig. 2. This electron micrograph shows, at higher magnification, an individual cell within the transplanted islet. Note its close proximity to an intra-islet capillary (R = red blood cell). The unique ultrastructure of its numerous insulin secretion granules (arrows) clearly identifies this cell type as a β-cell. ×6,446.

## DISCUSSION

The data reported here show that isolated pancreatic islets, transplanted simultaneously with SEF into the renal subcapsular space of MHC-incompatible donor rats, leads to protection of the islets against immunological destruction. Moreover, in the presence of a minimal amount of CsA, the data show that the rats are capable of developing a state of unresponsiveness or tolerance to their grafted islets that lasts indefinitely.

A number of mechanisms could be envisioned for the prolonged survival of islet allografts under these circumstances. First, despite the fact that special precautions were taken to transplant preparations consisting solely of Sertoli cells, these cells may, nonetheless, have been contaminated with either germ, or with Leydig cells. It has been demonstrated that sperm and seminal fluid suppress phytolectin-induced cell-mediated responses (13). Likewise, others have shown that Leydig cells nonspecifically inhibit lymphoproliferation, in vitro (14). We consider this an unlikely explanation, however, since our earlier studies have demonstrated that the destruction of either germ and/or Leydig cells in vivo does not prevent the long-term survival of intratesticular islet allografts (15), and because no such cell types were observed in the present study. Likewise, testicular steroidogenesis was shown not to be a prerequisite for the protection of intratesticular islet allografts against rejection (16).

Results suggest, rather, that the presence of the grafted Sertoli cells, per se, may have had an impact on the local immune response. Sertoli cells are responsible for the synthesis of many different protein substances, some of which promote growth (17,18), and others which have immunosuppressive capabilities (19). We have previously shown that cultured Sertoli cells produce a factor which inhibits Con-A stimulated lymphocyte proliferation in a dose-dependent manner (10). In this earlier study, lymphocyte proliferation was suppressed, and associated with an inhibition of the lymphokine interleukin-2 (IL-2). It is widely acknowledged that all proliferating T-cells express IL-2 receptors, while resting cells do not, and that interaction of IL-2 with its receptor is an absolute requirement for the clonal expansion of activated T-cells (20). Because the prevention of IL-2 receptor interaction completely inhibits T-cell proliferation, we propose that both clonal expansion and viability of activated T-cells are suppressed by an immuno-suppressive Sertoli cell secretory product (20).

If our premise is correct, then the most puzzling finding of the present study is the observation that transplantation of islets with SEF alone was not sufficient to protect the grafts against immunologic destruction. For successful transplantation, the grafted rats required an additional short course of CsA therapy. In effect, CsA proved to be a much more potent protector of the islets against rejection than did the SEF. For instance, in the control Group grafted with islets alone, but who were given CsA, graft survival occurred in 30% of the recipients (Table 1, Group 2). On


Fig. 3. This electron micrograph shows, at higher magnification, the fine structure of the extra-islet cells labeled "S" in Fig. 1. Both the cell nucleus (N) and cytoplasm are similar in appearance to Sertoli cells in vivo, and are structurally identical to Sertoli cells in vitro. R = red blood cell. ×3,280.

the other hand, in rats transplanted with islets and SEF alone, but without CsA, the grafts did not do nearly as well (Table 1, Group 3). In rats receiving both SEF and CsA, however, the cure rate was quite high, and about 70% of the grafted rats became normoglycemic for longer than 200 days (Table 1, Groups 4–6). We conclude from these findings that the simultaneous presence of SEF and CsA exerted synergistic effects essential for long-term islet allograft survival in the rat kidney.

At present we can only speculate on the mechanism(s) of this apparent synergistic effect. It is conceivable that the presence of SEF, or a factor produced by these cells, protected the islets against the toxic effects of CsA. It has been suggested that CsA may be directly toxic to β-cells (21,22). We regard this as an unlikely explanation. Results of a previous study have showed that β-cell function is not adversely affected in rats transplanted with islet xenografts which were treated with CsA for 100 days (7). Furthermore, in the present study the majority of rats were treated with only three injections of CsA, and this dose is unlikely to have been damaging to the islets.

A more likely explanation is that an immunosuppressive SEF secretory product, or products (20), enhanced, or augmented, the effects exerted by CsA on the immune response. CsA therapy is known to have a major impact on the immune response by means of several different mechanisms. First of all, CsA-induced effects result in a marked suppression of the production of the lymphokine, IL-2, and subsequently, a destruction of specific clones of proliferating cytotoxic T-cells (23–26). This effect would be similar to that caused by the proposed immunosuppressive Sertoli cell factor, as indicated in our earlier study (20). CsA has other modes of action, however, which are quite distinct from that mediated by the Sertoli cell. For example, CsA does not modify IL-2 receptor activity or expression, whereas the immunosuppressive Sertoli cell factor acts like a fusion protein to inhibit IL-2 receptor activity (20). More importantly, however, the treatment of grafted animals with CsA often leads to the induction of a state of specific tolerance or unresponsiveness to allografts (27–30).

Based on the results of this and our previous studies, we can now propose a likely mechanism for the synergistic effects of CsA and Sertoli cells on islet allograft survival: In the presence of both immunosuppressive components, there is a marked depletion of IL-2 production and blockage of IL-2 receptor activity resulting, therefore, in the ablation or significant reduction of proliferating cytotoxic T-cells. Prolongation of islet allograph survival is induced either by a clonal deletion of T-cells, or by the activation of a specific suppressor T-cell population (29,30). In this respect, it is of interest to note that others have induced a similar immune defect (i.e., suppression of rejection) for the protection of grafts such as the pancreatic Islet (31) and the heart (32) by treating recipient animals with a combination of an IL-2 antireceptor monoclonal antibody and CsA.

The importance of the present study relates to the likelihood that this approach to islet transplantation may also be applicable to the grafting of humans, and in particular, of both sexes. Aside from the effects of the immunosuppressive Sertoli cell factor on the immune response, it is encouraging to find that the effect occurred independently of the donor origin of the SEF. The results appeared to be the same whether the SEF donor was MHC-compatible with the host rat or not (Table 1, Groups 5 v. Group 6). The fact that SEF need not be MHC-compatible with the host greatly enhances the clinical applicability of this transplantation protocol. To this end, our investigation seeks to determine whether pre-incubation of Sertoli cells, prior to transplantation, alters the marked beneficial effects of the freshly collected SEF on islet allograft survival. Clearly, it would be more practical if cultured preparations could be used instead of dealing with primary cell isolation for each transplantation event. We are currently determining the success and efficacy of islet/SEF transplantation into other organ sites in both male and female rats.

In summary, we have shown that it is possible to create an immunologically privileged site in an organ other than the testis by means of the transplantation of SEF along with the islets. Indefinite islet allograft survival in the renal subcapsular space required, in addition, a short course of CsA therapy, although sustained immunosuppression was not essential. The data indicate that the SEF, and most likely Sertoli cells (10), retain the capacity to secrete an immunosuppressive factor(s) in an organ site other than the testis, and that the presence of these secretions were neither androgenic nor inhibitory to ovulation in female rats. This was demonstrated by the ability of grafted female rats to carry pregnancies to full term and to nurse their pups successfully.

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